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Quantitative considerations supporting the irrelevance of circulating serum CEA for the immunoscintigraphic visualization of CEA expressing carcinomas

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Abstract. Starting from the phenomenon that the amount of circulating CEA in patients' sera did not significantly influence immunoscintigraphic visualization of CEA expressing tumors, we built up an in vitro model to explain this phenomenon. Blocking experiments in this model system showed that the CEA specific MAbs BW 431/26 and BW 431/31 could not be inhibited in their binding to cell associated CEA, if they were preincubated with a 20 molar excess of serum CEA. In contrast, the CEA-NCA cross reactive MAbs could be inhibited in their binding to tumor associated CEA under identical conditions. These data combined with western blotting analysis of patients' sera and affinity constant determinations argue that conformational changes in serum CEA cause a decreased affinity of the CEA specific MAbs to serum CEA allowing a preferential binding to tumor associated CEA.

Key words: CEA – Conformational change - MAb – Immunoscintigraphy

Since the first description of carcinoembryonic antigen (CEA) (Gold and Freedman 1965a, b) until today, where the primary structure of CEA deduced from the cDNA sequence (Oikawa et al. 1987a) is known, CEA became important as a tumor marker for the treatment control of colorectal carcinomas (Egan et al. 1977; Fuks et al. 1980) as well as for the immunoscintigraphic visualization of CEA expressing carcinomas (Mach et al. 1981). In particular, the relatively new method of immunoscintigraphy demands monoclonal antibodies (MAb) binding to epitopes on CEA not detectable on the various nonspecific cross-reacting antigens (NCAs) expressed on normal tissues (von Kleist et al. 1972; Rogers 1983). A so called anti CEA antibody having strong cross-reactivity to human granulocytes and erythrocytes, i.e. NCA, was not able to localize human colorectal carcinomas in vivo, but induced fever, rigors, emesis and a short term granulocytopenia (Dillman et al. 1984). Also, those MAbs which are selective for protein epitopes on CEA (Buchegger et al. 1984; Bosslet et al. 1985) but not cross-reactive with epitopes on NCAs or Y or X type carbohydrate moieties on CEA (Nichols et al. 1985) are, after i.v. injection into the patient, primarily confronted with high amounts of circulating CEA. The following in vitro experiments try to explain why successful immunoscintigraphic localization of colorectal carcinomas using MAbs binding to CEA are possible with a sensitivity of up to 90% as shown in a multicenter study (100 patients) in the FRG using the MAb BW 431/31, coupled via DTPA to ^{111}In (Bosslet et al. 1987).

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Abbreviations used
CEA carcinoembryonic antigen; NCA nonspecific cross-reacting antigen; DTPA diethylene triamine pentaacetic acid; FBS fetal bovine serum; EDTA ethylene diamine tetraacetic acid; PCA perchloric acid; R.T. room temperature; PBS phosphate buffered saline; BSA bovine serum albumin; SDS-PAGE sodium dodecylsulfate polyacrylamide gel electrophoresis

Materials and methods

Tumor cell lines. The LoVo adenocarcinoma cell line of the colon was obtained from the American Type Culture Collection (ATCC, 12301 Parklawn Drive, Rockville, MD 20852, USA/ATCC-No.: CCL 229). It was routinely passaged in RPMI-1640 with 10% FBS. Confluent cultures were treated for 3 min with 0.25% trypsin 0.02 M EDTA and subcultured at a ratio of 1:5

Monoclonal antibodies. The MAbs BW 374/14, BW 250/183, BW 431/26 and BW431/31, all of IgG_1-isotype, recognize different epitopes on CEA (Bosslet et al. 1985, 1987). Briefly, the MAbs BW 431/26 and BW 431/31 are selective for CEA, whereas the MAbs BW 374/14 or BW 250/183 bind to NCA55 (=*n*on specific *c*ross-reacting *a*ntigen), NCA95 and CEA or NCA95 and CEA, respectively. MAb BW 494 binds to a pancreatic, gastric and colonic carcinoma associated mucin molecule different from CEA (Bosslet et al. 1986). MAb BW 575 is selective for small cell lung carcinomas and does not show any significant binding to gastrointestinal tract carcinomas (Bosslet 1987).

Preparation of peripheral blood granulocytes. Human peripheral blood granulocytes were isolated from heparin blood by treating the Ficoll-Hypaque pellet (Boyum 1976) for 5 min at 37° C with 0.87% NH_4Cl to lyse erythrocytes.

Preparation of granulocyte extracts. The erythrocyte depleted Ficoll-Hypaque pellet was solubilized by adding 0.5% Na-deoxycholate followed by 12 h head over head rotation at 4° C. After ultracentrifugation at 30000 g for 30 min at 4° C, aliquots of the supernatant corresponding to 2×10^5 granulocyte equivalents were transferred to individual lanes for SDS-PAGE and western blotting.

Radiolabelling of MAbs. Purified MAbs (Ey et al. 1978) were radiolabelled with ^{125}I using the iodogen method (Fraker and Speck 1978; Markwell and Fox 1978). MAb proteins were diluted in PBS containing 0.1% BSA (PBS-BSA).

The specific activities obtained for the different MAbs were:

BW 374/14:	3.9 μCi/μg,	BW 250/183:	3.5 μCi/μg
BW 431/31:	3.9 μCi/μg,	BW 431/26:	2.9 μCi/μg
BW 494:	4.03 μCi/μg,	BW 575:	2.59 μCi/μg

Blocking assay. ^{125}I labelled MAb protein (100 μl) diluted in PBS-BSA was mixed with CEA containing serum (100 μl) or purified CEA isolated from CEA expressing human colon carcinomas and incubated for 90 min at 37° C in round bottom 96 well microtiter plates (Nunc: 1-63320). Thereafter, 10^6 LoVo cells in 25 μl PBS-BSA which were detached from plastic by incubation with 0.02% EDTA for 5 min, were added to the MAb serum or MAb-CEA mixture. After 1 h incubation at 37° C, the microtiter plates were spun and the pellets were washed 3 times with PBS. The washed cell pellet was resuspended in 125 μl and 100 μl thereof and counted in a well type γ-counter to determine the cell bound radioactivity.

Western blotting analysis. The methodology was performed essentially as described by Towbin and Gordon (1979), but instead of radiolabelled second antibody, the ABC vector kit (Vector Burlingname) was used.

Scatchard analysis. The affinity of the MAbs to tumor cells was evaluated according to Walker (1977), based on the original papers of Scatchard (1949) and Berson and Yalow (1959). As antigenic material collagenized and formaldehyde fixed cells derived from nude mouse xenografts transplanted with the CEA expressing CoCa[2] colonic carcinoma were used.

A constant amount of these cells is added to each vial of a dilution series of the respective MAb. This mixture is incubated for 4 h at room temperature, the cells were spun down, washed and the cell bound activity determined.

The bound to free ratio is plotted against concentration of cell bound antibody molecules using the following corrections (Steinsträsser et al. 1987): (1) The unspecific bound part (U) of antibodies (determined with irrelevant cells or by an iteration procedure during the evaluation) is subtracted. (2) The free part of antibodies (F) consists only of immune reactive molecules using the formula $F = r \times T - (B - U)$, where T is the total activity and r the immune reactive fraction of the preparation, determined separately according to Steinsträsser and Schwarz (1986).

The inverse of the slope of the described plot gives the affinity constant (l/mol).

CEA assay. The amount of CEA present in patients' sera was evaluated using the commercially available Behring Enzygnost CEA assay.

Results

Binding inhibition experiments on LoVo cells

Incubation of 10^6 CEA expressing living LoVo colon carcinoma cells with 2–3 ng (10000 cpm) of the various MAb proteins for 60 min at 37° C results in the binding of 0.2 0.3 ng (1000 cpm) to the membrane of the cells. Preincubation of the various MAbs (≈10000 cpm) for 90 min at 37° C with colon carcinoma patients' sera (Fig. 1) containing high levels (a–d), medium levels (e–f), or low levels (g–j) of CEA had the following effects on MAb binding to the cells (Fig. 1):

The binding of MAbs BW 431/26 and BW 431/31 to the LoVo cells is only weakly inhibited despite a 20 fold molar excess of soluble CEA (a–d). In contrast, the binding of MAbs BW 250/183 and BW 374/14 to the LoVo cells is efficiently inhibited under identical conditions (a–d). Marginal binding inhibition was observed with all anti CEA MAbs with medium (e–f) or low (g–j) CEA containing sera.

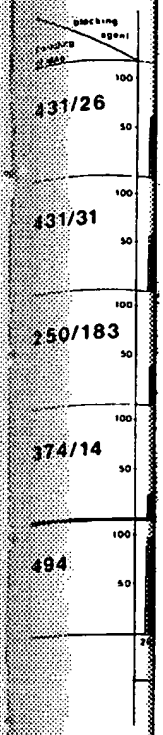
Purified CEA (Krupey et al. 1968) isolated from a colon carcinoma primary tumor did not inhibit the binding of MAbs BW 431/26 and BW 431/31 to the LoVo cells as efficiently as it inhibited the binding of MAbs BW 374/14 and BW 250/183 (k).

The positive control MAb BW 494 binding to a high molecular weight mucin epitope (Bosslet et al. 1986) not detectable on CEA could not be inhibited in its binding to the LoVo cells, neither by CEA containing sera (a–j) nor by purified CEA (k). The binding of the anti small cell lung carcinoma MAb BW 575 (Bosslet et al. 1987) was in the range 1%–3% of the binding obtained with the anti CEA MAbs or MAb BW 494 (data not shown). Purified human serum albumin (l) as well as phosphate buffered saline (m) showed no inhibition of binding with any MAb.

In summary, the binding of the anti CEA-NCA MAbs BW 374/14 and BW 250/183 is equally effectively inhibited by CEA containing sera and purified CEA, whereas the binding of the anti CEA MAbs BW 431/26 and BW 431/31 is neither inhibited by the purified CEA isolated from a primary colonic carcinoma nor by the CEA containing sera

Western blotting analysis of CEA containing patient's sera

To investigate whether the reduced inhibition mediated by the CEA containing sera on the binding of the CEA specific MAbs BW 431/26 and BW 431/31 to the LoVo colonic carcinoma cell line is due to the absence of intact CEA in the sera, we performed a western blotting analysis after perchloric acid (PCA) extraction with the same sera and CEA preparation as used in the preceding inhibition experiments. The data presented in Fig. 2A, B, C, D show that all four MAbs bound to purified CEA attached to nitrocellulose filter (1 μg) to a similar extent, visualizing a 180 kDa band (lane k, Fig. 2, A, B, C, D). The CEA specific MAbs BW 431/26 and BW 431/31 did not bind to PCA extracts from colonic carcinoma patients' sera (Fig. 2A, B, lane a, b, c, d), whereas the CEA-NCA cross-reactive MAbs visualize the 180 kDa CEA band (Fig. 2, C, D lane a, b, c, d). Neither MAb bound to PCA extracts of a control serum (Fig. 2A, B, C, D lane g). As a positive control for the binding potential of MAbs BW 374/14 and BW 250/183 to NCA95, they were reacted with Na-deoxycholate extracts from human peripheral blood granulocytes. In lane o, Fig. 2C, D, the NCA band was significantly stained, where-



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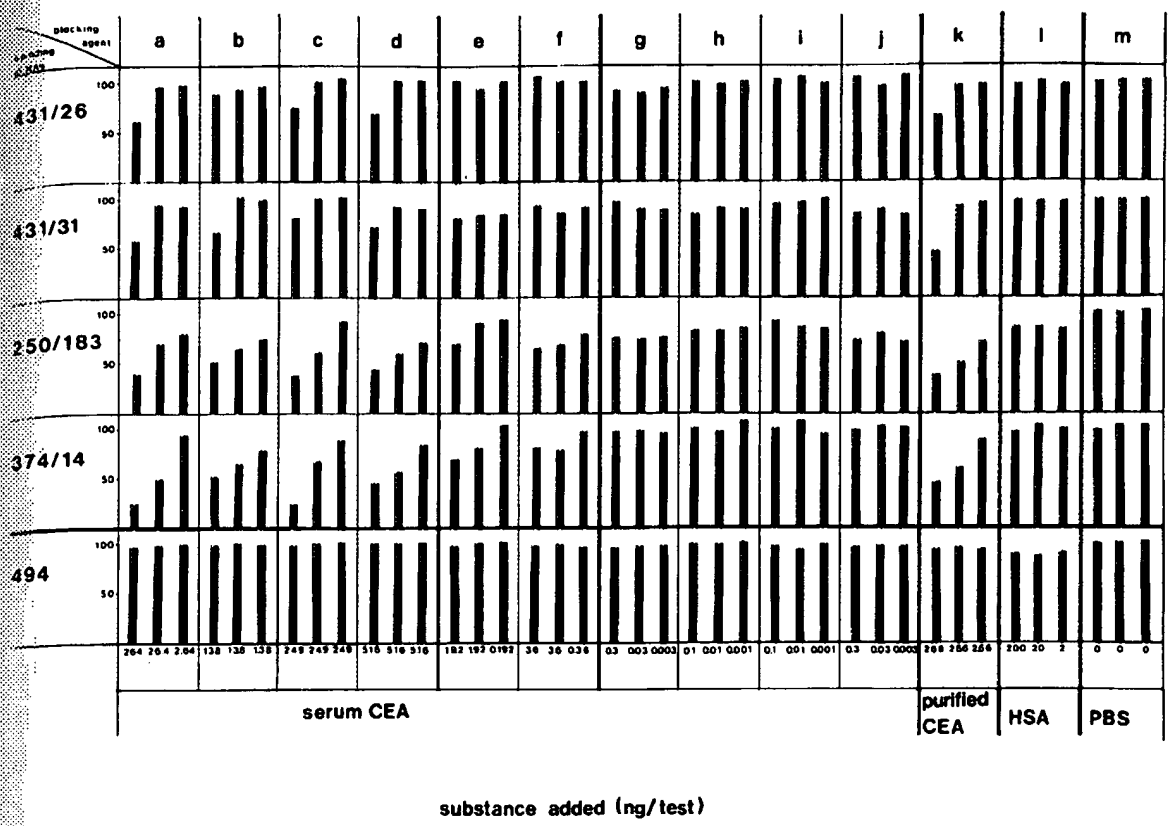

Fig. 1 a–m. In the binding inhibition experiment black columns represent the percentage of the respective MAbs bound to cell associated CEA after blocking with sera from patients with high (**a–d**), medium (**e, f**) or low (**g–j**) CEA content in comparison to purified swimming CEA (**k**), HSA (**l**) or PBS (**m**)

as the 2 CEA specific MAbs BW 431/26 and BW 431/31 did not show any binding to granulocyte NCA (lane o, Fig. 2 A, B).

In summary, these data indicate that colonic carcinoma patients' sera contained intact 180 kDa CEA which was detected by the CEA-NCA cross-reactive MAbs, but not by the CEA specific MAbs. No NCA could be detected in patients' sera by neither MAb. But granulocyte associated NCA95 (Buchegger et al. 1984) was detected by MAbs BW 374/14 and BW 250/183. In contrast purified CEA from a colonic carcinoma primary tumor was detected to a similar extent by the four specific MAbs used. The irrelevant MAbs BW 494 and BW 575 did not show any binding to purified CEA or CEA containing patients' sera (data not shown).

Estimation of the in vitro binding affinity of MAbs to cell membrane bound CEA

To exclude that simple differences in the affinity of the various MAbs to CEA were responsible for the results observed in the binding inhibition and western blotting experiments, the affinity constants of the MAbs to tissue associated CEA were determined as detailed in Material and Methods. As antigen source, collagenized, formaldehyde fixed cells from colon carcinoma nude mouse xenografts were used. The affinity constants calculated are shown in Table 1.

Original scatchard plots from a representative experiment are shown in Fig. 3 for MAb BW 431/26 (A), BW 431/31 (B) and BW 250/183 (C). In Table 1 affinity constants evaluated from various experiments are given, supporting the medium affinity constant values mentioned above.

Discussion

The paper deals with the so far unexplained phenomenon that CEA specific MAbs are able to localize CEA expressing tumors in the patient despite significant amounts of circulating CEA in the blood. In the binding inhibition experiments we could show that MAbs directed to CEA specific epitopes on CEA (MAb BW 431/26 and BW 431/31) are only marginally blocked by CEA containing sera. The CEA content of these sera was in 20fold molar excess (500 ng CEA↔2 ng MAb) compared to the radiolabelled MAbs used in the binding assays.

In contrast, the CEA-NCA cross-reactive MAbs could be more efficiently blocked under identical conditions. These findings could be explained by the presence of NCA in the CEA containing sera mediating the more efficient blocking of the CEA-NCA cross-reactive MAbs, or by higher affinities of the CEA-NCA MAbs to circulating CEA. The western blotting analysis performed thereafter showed that the CEA containing sera did not contain detectable amounts of NCA, arguing against the first explanation. In contrast, NCA from normal human granulocytes

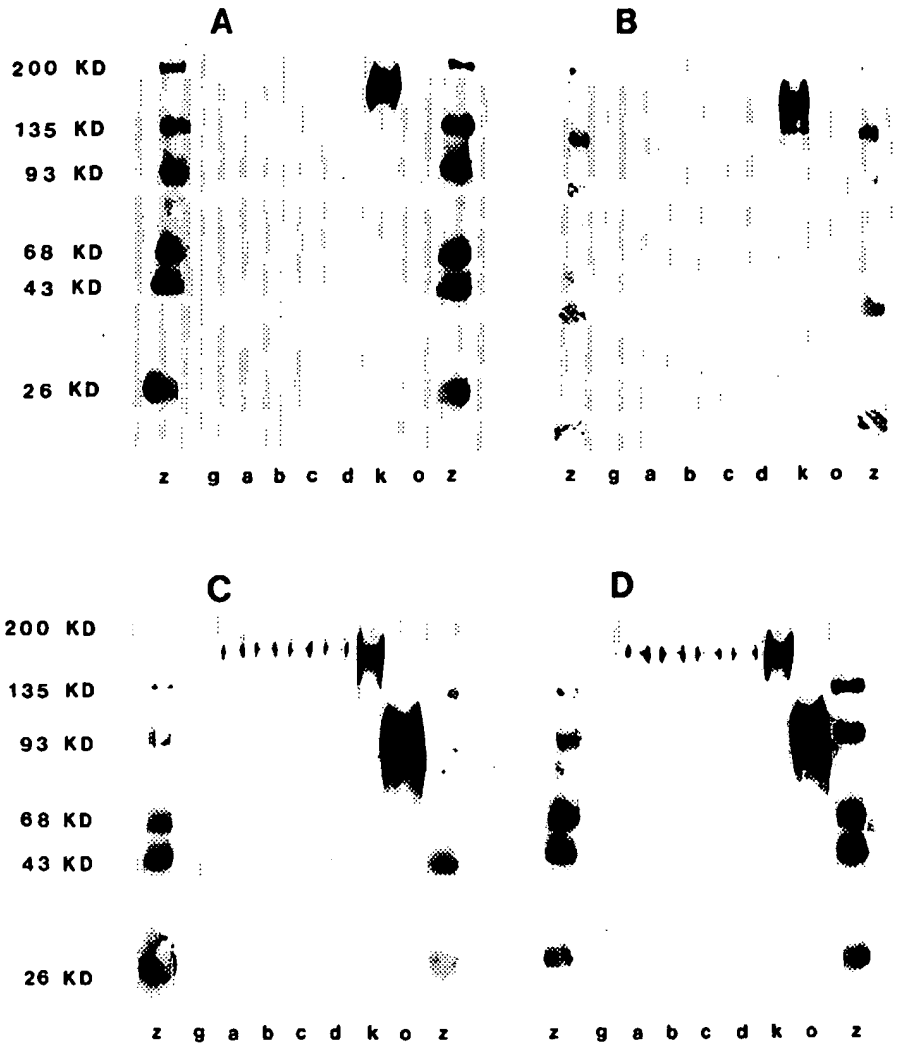

Fig. 2. Photograph of 4 blots independently blotted by means of 10% SDS-PAGES immunostained with **A** MAb BW 431/26, **B** MAb BW 431/31, **C** MAb BW 250/183, **D** MAb BW 374/14. Lanes a–d represent PCA extracts from patients sera containing CEA (compare Fig. 1), lane *g* represents control serum, lane *k* represents purified CEA, lane *o* represents Na-deoxycholate extracts from normal human granulocytes (2×10^5 granulocyte equivalents/lane). Lane *z* visualizes the rainbow protein molecular weight markers (Amersham)

Table 1. Determination of affinity-constant by scatchard evaluation

MAb	Number of determinations	Ka ± sd	range
431/31	17	$3 \pm 2 \times 10^8$	$1 \times 10^8 \ldots 8 \times 10^8$
250/183	6	$2 \pm 1 \times 10^9$	$1 \times 10^9 \ldots 4 \times 10^9$
431/26	5	$9 \pm 12 \times 10^9$	$1 \times 10^9 \ldots 3 \times 10^{10}$

could be detected under the same experimental conditions with MAbs BW 250/183 and BW 374/14. Furthermore, the western blot data indicated that the CEA-specific MAbs (BW 431/26 and BW 431/31) as well as the CEA-NCA cross-reactive MAbs (BW 250/183 and BW 374/14) were able to detect CEA purified from primary tumors attached to nitrocellulose filter to a similar extent.

Therefore, the second explanation that the CEA-NCA cross-reactive MAbs have a higher affinity to circulating CEA than the CEA-specific ones is probably correct. The scatchard plot data showed that the CEA-specific MAb BW 431/26 had the highest affinity (in the range 10^{10} l/mol) if cell bound CEA is used as epitope bearing antigen. The reduced binding, especially of this MAb to swimming CEA as evidenced by the binding inhibition experiments could be explained by differences in the conformation between cell bound and circulating CEA. Especially the class five epitopes (Kuroki et al. 1987) which are unique to CEA are known to be weakly immunogenic if purified CEA is used as immunogen. Furthermore, the affinity of those MAbs to CEA is mostly in the range of 10^8 l/mol, but MAb BW 431/26, having an affinity constant of 9×10^9 l/mol, is an exception allowing successful immunoscintigrams of colorectal carcinomas 4–6 h after i.v. application of the ^{99m}Tc-MAb-immunoconjugate in the patient (A. Schwarz manuscript in preparation). The success of the immunoscintigraphic evaluation is independent of the CEA content in the patients' serum as revealed in a multicenter study performed with MAb BW 431/31 (Bosslet et al. 1987).

This clinical finding is mimiced by our in vitro binding inhibition model. We confronted our radiolabelled MAbs for 90 min with up to 500 ng (1.6×10^{12} molecules) CEA from CEA containing sera. This is a 20fold molar excess compared to the 2 ng of MAb (8×10^{10} molecules). There-

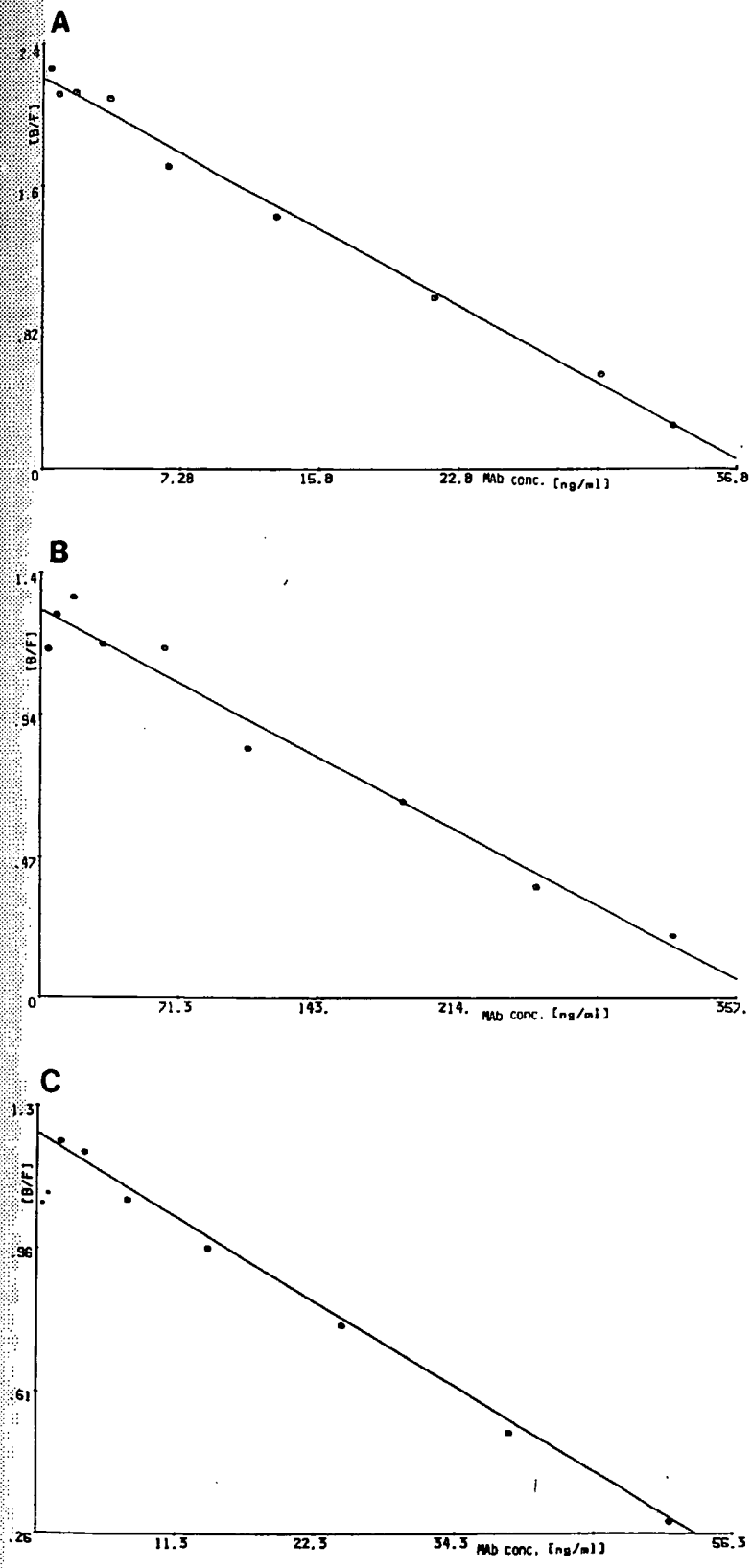

Fig. 3A–C. Scatchard plots are depicted where the bound (B) to free ratio (F) was plotted against the cell bound MAb concentration. A–C represent plots obtained for MAbs BW 431/26, BW 431/31 and BW 250/183 respectively

after, to this mixture 10^6 LoVo cells were added bearing between 10^{10}–10^{11} CEA epitopes (10^4–10^5 epitopes/cell) and incubated for another h. The binding data showed that especially the CEA specific MAbs BW 431/26 and BW 431/31 bound very efficiently to the CEA expressing cells arguing for a preferential binding of these MAbs to cell bound CEA. For scintigraphic purposes with anti CEA MAbs, patients receive 2 mg of purified MAb resulting in a concentration of 670 ng MAb/ml (2.7×10^{12} molecules/ml). The serum CEA concentrations in colonic carcinoma patients are mostly in the range 1–3 ng CEA/ml (maximally 1×10^{13} molecules/ml) yielding maximally a 4fold molar excess of CEA compared to the MAb in the serum. This molar excess of circulating CEA does not seem to be high enough to block the binding of the MAb to the tumor associated CEA epitopes. Despite our detailed knowledge about CEA, i.e. the cDNA sequence (Oikawa et al. 1987a), the immunoglobulin domain like structure (Oikawa et al. 1987b), the N linked carbohydrate chains (Chandrasekaran et al. 1983) and the NH_2 terminal amino acid sequence (Engvall et al. 1978), the exact structure of MAb defined epitopes as well as the MAb detected conformational differences between serum CEA and tumor associated CEA remain to be elucidated.

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